Розглянуто можливість збільшення біомаси Chlorella vulgaris та вмісту в клітинах ліпідної фракції за допомогою звукового опромінення. Показано, що найбільший приріст біомаси і ліпідної фракції спостерігається при опроміненні ультразвуком частотою 20 кГц. Опромінення нижчими частотами призводить до зниження приросту біомаси, але підвищує питомий вміст ліпідної фракції

Ключові слова: культивування, мікроводорості, опромінення звуковими частотами, ліпіди, ультразвук, Chlorella vulgaris, біомаса

Рассмотрена возможность увеличения биомассы Chlorella vulgaris и содержания в клетках липидной фракции с помощью звукового облучения. Показано, что наибольший прирост биомассы и липидной фракции наблюдается при облучении частотой 20 кГц. Облучение низкими частотами приводит к понижению прироста биомассы, но повышению удельного содержания липидной фракции

Ключевые слова: культивирование, микроводоросли, облучение звуковыми частотами, липиды, ультразвук, Chlorella vulgaris, биомасса

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IMPACT OF SOUND IRRADIATION ON CHLORELLA VULGARIS CELL METABOLISM

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1. Introduction

Ukraine and other countries face the problem of substituting traditional energy resources with alternative clean fuel. One of these sources is biodiesel, which is produced from the microalgae lipid fraction. The problem of microalgae cultivation for biodiesel production is that the maximum biomass growth occurs under optimum cultivation conditions while the accumulation of cell lipids, namely triacylglycerols – under stress conditions. These conditions are created mostly by changing the culture medium structure (lack of nitrogen or sulfur), or process parameters (light, pH, etc.) [1]. Therefore, the actual problem is to study the stress factors, which lead to a simultaneous increase in the rate of microalgae biomass growth and lipid fraction accumulation.

2. Literature review and problem statement

One factor that affects the cell metabolism of microorganisms is irradiation of different frequencies. It is known that ultrasound irradiation has a stimulating effect on representatives of the plant and fungal kingdoms [2]. Thus, ultrasound pretreatment of *Saccharomyces vini* yeast cells, the sound energy density of $15{\cdot}10^3$ – $20{\cdot}10^3$ kJ/m^3, a frequency of 22 kHz for 3–5 minutes stimulates the biomass growth by 2.8–3 times [3]. The effect of low–frequency ultrasound irradiation of 20–30 kHz on the *Saccharomyces cerevisiae* M30 yeast culture leads to increased fermentation of sugars and high amount of ethanol, resulting from fermentation by 4.6 % when irradiated with ultrasound at a frequency of 25 kHz [4].

Nihal E. Al-Taee and coauthors have found that the use of ultrasound in the treatment of seeds and cells increases the enzymatic activity of enzymes such as thymidylate synthase, dihydrofolate reductase, hydroxymethyltransferase, and the synthesis of nucleic acids and proteins in cells of higher plants (sesame). Using a frequency of 50 kHz for 40 minutes raises the protein synthesis almost by 5 times, the RNA and DNA contents – almost by 10 times, the callus cell biomass – by 3 times [5]. The ultrasound irradiation also accelerates the development of plant tissues. For example, the growth rate of both shoot and root of carrot seeds raises by 15 % (22 kHz, power 150 W, 3 min) [6].

Sound spectrum irradiation also has a stimulating effect on the cells of higher plants [7–9]. Thus, irradiation with a frequency of 0.1–2 kHz, loudness of 100 dB increases the permeability of membranes, improves the activity of protective enzymes and cell division rate. The biomass growth raises by 6–30 % depending on the frequency applied [7, 8]. In [8, 9], it is found that both sound and ultrasound irradiation increase the active transport rate in plant cells as a result of increased activity of membrane H^+–ATPase under the high content of Ca^{2+} ions in the cytoplasm.

For each plant, there is a threshold of sensitivity to sound vibrations in the range of 100 Hz – 30 kHz, with the membrane destruction and cell death which may occur upon reaching it. For most plants increased frequency leads to significant changes in the cells [10]. It is, therefore, necessary to establish the irradiation duration, which stimulates development and does not cause cell death.

In the microalgae cultivation, irradiation with different frequencies (40–1146 kHz) was mainly used for cell disintegration and lipid fraction isolation [11–13]. Irradiation with frequencies of 20 kHz (600 W) and 200 kHz (100 W) for the cultivation of *Microcystis aeruginosa* UTEX 2388 cyanobacteria increases the cell size, content of chlorophyll and lipid fraction [14]. In [15], it is shown that low-power ultrasound irradiation raises the biosynthesis of polyunsaturated fatty acids in microalgae.

Based on the above, we can conclude that the effect of sound and ultrasound irradiation on microalgae cells may alter the cell metabolism, resulting in the increased biosynthesis of triacylglycerols – feedstock for biodiesel production. So, the study of the effect of irradiation with different frequencies on *Chlorella vulgaris* cell metabolism is an urgent problem.

3. Research purpose and objectives

The purpose of the paper is to determine the impact of sound irradiation on the metabolism and biomass growth of *Chlorella vulgaris* to be used as a bioenergy feedstock.

To achieve this purpose, the following tasks are solved:

– to scientifically prove and experimentally confirm the impact of sound irradiation of different frequencies on the growth and development of *Chlorella vulgaris* microalgae;

– to examine the changes in *Chlorella vulgaris* cell metabolism and lipid fraction yield under irradiation of different frequencies.

4. Materials and methods of investigating the impact of irradiation of different frequencies on the *Chlorella vulgaris* microalgae culture

4. 1. Investigated materials and equipment used in the experiment

The study was conducted using *Chlorella vulgaris* ACKU531–06 microalgae from the collection of the Taras Shevchenko National University of Kyiv (Ukraine).

Microalgae cultivation was carried out in the 1.3 dm^3 photoreactors at a temperature of 18±2°. Provision of cells with light energy was performed using natural light and combined lamps with red and blue LEDs. Such lighting is defined suitable for cultivation [16].

The Gromov 6 medium, prepared by a standard method served as culture medium [17]. The medium was autoclaved for 1 hour at a temperature of 120° and a pressure of 250 kPa. Bubbling of CO_2 medium for 1 minute at a rate of 1 $dm^3/(min{\cdot}dm^3)$ was conducted every 24 hours.

The *Chlorella vulgaris* inoculant, introduced in the photoreactor was 20 % of the tank volume. The initial absorbancy of D_{450} suspension was 0.375±0.01, corresponding to the content of 8160000±217600 cells in cm^3.

Irradiation of the suspension was carried out using the GM800K square wave generator and piezo element.

Monitoring and control of the *Chlorella vulgaris* culture purity were performed by light microscopy using a microscope TM XSP–139TP (Ulab, China) (zoom from 40x to 1000x for visual observation, from 4x to 1000x to camera shooting).

The vacuum filtration at the vacuum filtration device PVF–35(47)/1 VN (Russia) was used for the biomass separation from the culture medium.

4. 2. Methodology of culture irradiation and methods for determining the biomass growth and lipid fraction content

The frequencies of 20 kHz, 15 kHz, 10 kHz, 5 kHz were selected to determine the impact of sound vibrations on the microalgae cell metabolism. The irradiator power was identical for all frequencies and amounted to 5 W/cm^2. The control sample was not irradiated. Irradiation was carried out 1 time a day for 1 min.

Counting of microalgae cells was performed in the Goryaev chamber by the standard method [18] using the formula:

$$X = (a \cdot 4000 \cdot y)/b \text{ , cells/}\mu l, \quad (1)$$

where X is the number of cells in the suspension, in 1 mm^3; a is the number of cells in the suspension, counted in a certain volume of the chamber; b is the number of counted small squares; y is the suspension dilution. Counting was performed at a low microscope magnification (lens 8x, eyepiece 10x).

The study of the biomass growth dynamics was performed by measuring the absorbancy of cultures at ULAB 102 spectrophotometer at a wavelength of 450 nm (D_{450}), since the maximum light absorption spectrum of chlorophyll a is in the range of 430÷450 nm [19]. For the study, glass cuvettes with an optical path length of l=1 cm were used. The biomass growth was found in the calibration curve.

Isolation of *Chlorella vulgaris* cells from the culture medium was conducted by filtering out through the "blue ribbon" filter, which was previously weighed, by a vacuum pump. The precipitate was dried in the drying oven 2B–151 (the RF) at 105 °C to constant weight. The biomass concentration was determined by the formula:

$$C = \frac{(m_k - m_n) \cdot 1000}{V} \text{ , g/dm}^3, \quad (2)$$

where $m_к$ is the weight of the filter with the biomass after drying; $m_п$ is the filter weight, 1000 is the equivalent volume of dm^3, V is the aliquot to produce dry biomass.

The specific growth rate of microalgae cells μ per day was calculated by the formula (3):

$$\mu = (\ln N_t - \ln N_0)/t, \text{ day}^{-1}, \quad (3)$$

where N_t is the number of microalgae cells per unit volume at time t; N_0 is the number of microalgae cells per unit volume at the beginning of cultivation; t is the cultivation duration, days.

Determination of the lipid fraction content was carried out according to GOST 13496.15–97 using the Soxhlet method [20]. The sample of microalgae dry mass was placed in the Soxhlet apparatus (Ulab). Hexane was used as an extractant. After extraction, the solvent was distilled off and the flask with lipids was dried to constant weight. The lipid weight was calculated by the weight difference between the flask with lipids and an empty flask. According to the method, maximum deviation does not exceed 1 %.

5. Results of investigating the impact of sound irradiation on the development and accumulation of *Chlorella vulgaris* lipid fraction

The dynamics of *Chlorella vulgaris* biomass growth under irradiation by sound waves of different frequencies and start of the ultrasound spectrum (20 kHz) are shown in Fig. 1. As shown in Figure, a slow growth phase for all samples lasts for 6 days. It is followed by the exponential growth phase at frequencies of 20 kHz and 15 kHz for 8 days. At lower frequencies – 10 kHz and 5 kHz, the culture growth rate

is slower, but lasts for 14–15 days, with a slightly greater biomass growth observed in the culture, irradiated at a frequency of 10 kHz. The biomass growth of the culture, which was not exposed to irradiation (control) increases compared to the cultures irradiated at low frequencies after the 20th day of cultivation. Achieving a constant absorbancy of all cultures is due to the colony formation and fast settling of cells during the absorbancy measurement.

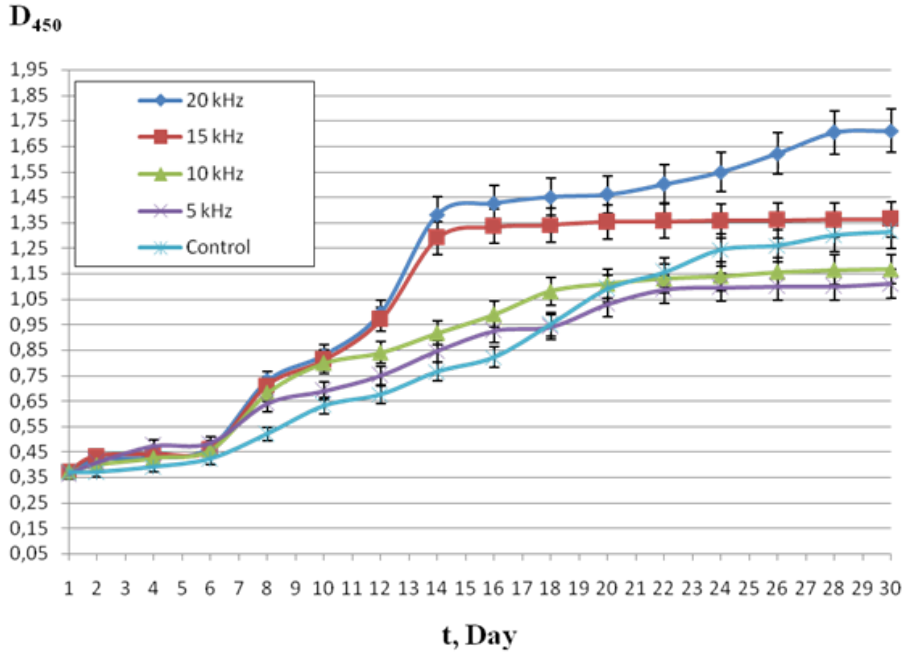

Fig. 1. The change in absorbancy (D_{450}) of the *Chlorella vulgaris* culture in the cultivation process (t) under irradiation with the sound vibrations of different frequencies: 5, 10, 15, 20 kHz

The change in the growth rate of *Chlorella vulgaris* cells under irradiation with different frequencies is shown in Fig. 2. The effect of all wavelengths leads to an intense increase in the relative cell growth starting from the 4th day and lasts 4 days for high frequencies (15 and 20 kHz) and 6 days for low (5 and 10 kHz). The relative cell growth in the control exceeds the cell growth under the low-frequency irradiation after the 11th day.

Sound irradiation of different frequencies affects the cell shape and colony formation. When cultured under sound waves, the cell size on average increases with growing irradiation frequency and reach (5–10 μm) compared with the control (2–6 μm). The maximum cell size is characteristic of the ultrasound treatment (20 kHz). Also, in the absence of irradiation treatment, cells form large colonies and cultivation of individual cells is hardly observed on the 7–8th day. When treated with sound waves, the colonies are small and have irregular edges, with increased content of single cells.

Fig. 3 shows the growth of biomass and lipids within 30 days of cultivation under different frequency irradiation. As shown in Fig. 3, irradiation affects the biomass growth and lipid fraction yield. The largest growth is observed under irradiation at a frequency of 20 kHz, with the biomass growth exceeding the weight of microalgae, grown without irradiation by 10 %. Based on Fig. 1, 2, the microalgae growth under irradiation occurs at a higher rate. Thus, under ultrasound irradiation, the biomass growth rate is 1.6 times greater than in the control sample and, therefore, the cultivation period in the industrial production can be reduced. Irradiation at frequencies of 10 and 15 kHz reduces the biomass growth by 10 % compared with the control experiment, despite the high content of chlorophyll *a*, as evidenced by a greater absorbancy of the culture (Fig. 1). The growth is almost identical for these frequencies and is within the experimental error. Cultivation under a frequency of 5 kHz leads to a slight increase in the biomass yield.

Cultivation of *Chlorella vulgaris* microalgae under irradiation with different frequencies leads to changes in the cell metabolism and increase in the lipid fraction biosynthesis. So, the lipid content increases by 3 times compared to the control sample for the culture grown under ultrasound irradiation (20 kHz). Under the 5 kHz irradiation, the lipid content is doubled. The minimum growth of lipids and biomass is observed under 15 kHz irradiation.

Fig. 4 shows the specific lipid content in the dry biomass of microalgae depending on the irradiation frequency. The impact of ultrasound irradiation (20 kHz) leads to raised triacylglycerol biosynthesis and, consequently, maximum specific content.

The irradiation frequency of 10 kHz, although reduces the growth rate, but increases the specific content of the lipid fraction by 2 times compared to the control. The lowest lipid growth is in the culture, grown under irradiation at 15 kHz. Though the lipid content is 56 % higher compared to the control, the biomass growth reduction by 10 % suggests the use of such frequency inappropriate in the microalgae cultivation for biodiesel production.

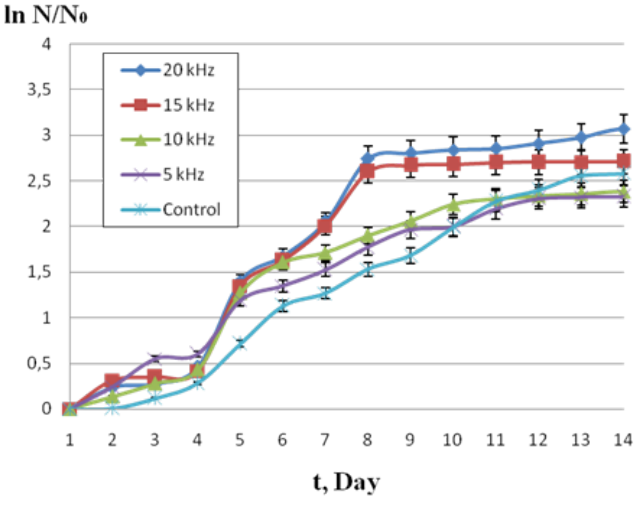

Fig. 2. The relative growth of *Chlorella vulgaris* cells ($\ln N/N_0$) over time (t) under the influence of sound vibrations with frequencies of 5, 10, 15, 20 kHz

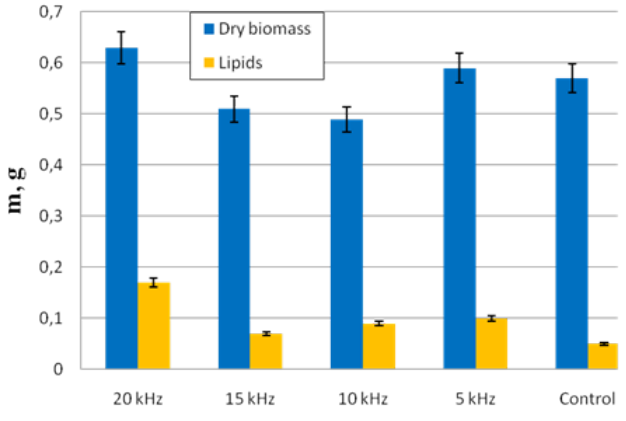

Fig. 3. The growth of biomass and lipids of *Chlorella vulgaris* microalgae (m) under the influence of sound vibrations with frequencies of 5, 10, 15, 20 kHz compared to the unirradiated culture (control)

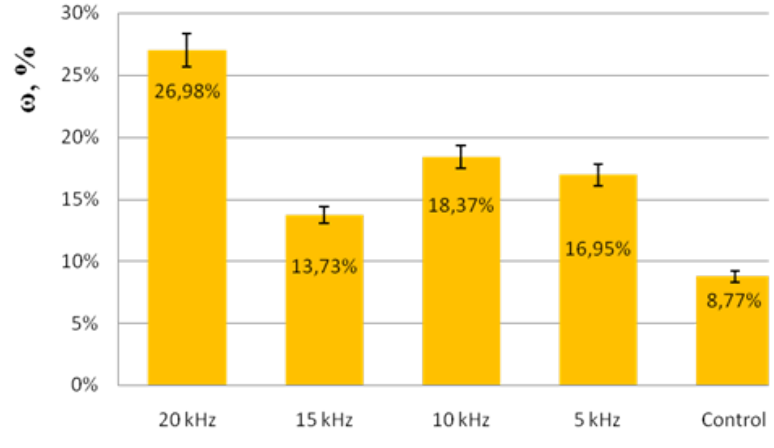

Fig. 4. The specific content of the lipid fraction (ω) in *Chlorella vulgaris* depending on the impact of sound vibrations with frequencies of 5, 10, 15, 20 kHz compared to the control experiment

trasound intensifies the mass transfer through membranes due to their depolarization and microcirculation of organic and inorganic compounds, which raises the active transport of nutrients through the membrane [2]. Ultrasound also affects the deposition rate of cells on the photoreactor surface. Ultrasound leads to the destruction of colonies, detachment of cells from the reactor walls and, consequently, reduction of the areas of shading and enhanced lighting of the culture. Low biomass growth in relation to the data obtained in [21] is due to the cultivation process at low temperatures, which are different from optimum for *Chlorella vulgaris*.

It is the size of cells and their grouping that cause differences in the biomass growth rate and absorbancy indicators of the suspension (Fig. 1, 3) when exposed to the sound frequency of 15 kHz. That is, irradiation increases the biosynthesis of pigments of the photosynthetic system, but the proportional biomass growth does not occur. This reduces both biomass growth and accumulation of lipids compared to the culture, irradiated with a frequency of 20 kHz. This effect of sound irradiation at 15 and 10 kHz can be attributed to their destructive effect on cell membranes. Probably, constant action of such frequencies destroys weak links through increased oscillation amplitude of the lipoprotein atoms, leading to partial cell death, as evidenced by the absence of growth after 14 and 18 days for the given frequencies, respectively (Fig. 1).

Sound irradiation with a frequency of 5 kHz, on the contrary, has a stimulating effect, accelerates active transport due to increased activity of the membrane H^+–ATPase, leading to the high growth of the lipid fraction and biomass as a whole compared to the control sample.

6. Discussion of results of the impact of sound irradiation on *Chlorella vulgaris* microalgae cell metabolism

Since all cultivation conditions were identical except for irradiation with sound waves of different frequencies, based on these data it can be argued that irradiation with frequencies of 5, 10, 15 and 20 kHz affects the intensity and direction of microalgae metabolism.

These data suggest that the effect of sound irradiation of different frequencies on the microalgae cell metabolism is ambiguous. Under the influence of any frequency, cells begin to increase the biosynthesis and accumulate triacylglycerols – feedstock for biodiesel production. It means that irradiation is a stress factor for cells, resulting in the raised biosynthesis of substances that serve as energy reserves, for *Chlorella vulgaris* cells these are lipids (Fig. 3, 4).

The development of cells is the most intensively affected by ultrasound irradiation (20 kHz). This frequency increases the chlorophyll biosynthesis, and, therefore, the photosynthesis intensity, as evidenced by the angle on the exponential curve of absorbancy (Fig. 1). This increases the biomass accumulation rate through the lipid fraction formation, as evidenced by increased size of the cells. Ul-

7. Conclusions

1. The effect of sound irradiation of different frequencies on the *Chlorella vulgaris* biomass growth and biosynthesis of lipids – feedstock for biodiesel production is shown. Irradiation of microalgae cells with frequencies of 10 and 15 kHz increases the biosynthesis of pigments of the photosynthetic system, but inhibits the biosynthetic processes, reducing the biomass growth. Irradiation with a frequency of 5 kHz has no inhibitory effect, and the biomass growth is equal to the growth without irradiation. Ultrasound irradiation with a frequency of 20 kHz intensifies active transport of compounds and ions through the membrane and raises the cell metabolism, which accelerates biosynthetic processes and increases the biomass growth by 10 %.

2. It is found that sound irradiation with frequencies of 5, 10, 15 and 20 kHz increases the synthesis of triacylglycerols. The specific content of the lipid fraction exceeds its content in non-irradiated cells by 1.5, 2.1 and 2 times for the frequencies of 15, 10 and 5 kHz, respectively. Ultrasound irradiation with a frequency of 20 kHz increases lipid biosynthesis by 3 times compared to non-irradiated cells. This irradiation frequency is optimum among the studied frequencies to be used as a factor of influence for biodiesel production from *Chlorella vulgaris* microalgae.

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